Na,K-ATPase as a Signal Transducer

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ABSTRACT: Recent studies have indicated that Na,K-ATPase may, in addition to being the key regulator of intracellular Na^+ and K^+ concentration, act as a signal transducer. Despite extensive research, the biological role for ouabain, a natural ligand of Na,K-ATPase, is not well understood. We have reported that exposure of rat proximal tubular cells (RPTC) to doses of ouabain that inhibit the Na,K-ATPase activity by less than 50% (10 nM – 500 μM), will induce intracellular $[Ca^{2+}]_i$ oscillations and that this calcium signal leads to activation of the transcription factor NF-κB. The ouabain-induced calcium oscillations were blocked by an inhibitor of the IP_3 receptors but not by phospholipase C inhibitors nor by cellular depletion of IP_3, suggesting that the calcium signal is not due to phospholipase C–mediated IP_3 release. Fluorescence resonance energy transfer (FRET) studies suggested a close proximity between the Na,K-ATPase and IP_3 receptor. Our findings demonstrate a novel principle for calcium signaling via Na,K-ATPase.

KEYWORDS: Na,K-ATPase; Ca^{2+} oscillations; ouabain; IP_3 receptor; FRET; transcriptional regulation; NF-κB

INTRODUCTION

Several recent studies suggest that in addition to its key role as a regulator of cell ion homeostasis, Na,K-ATPase may act as a signal transducer and activator of gene transcription.[1–4] To further elucidate this role for Na,K-ATPase, we have investigated intracellular signaling pathways activated by the ouabain/Na,K-ATPase complex. Here we report that ouabain-bound Na,K-ATPase can induce intracellular calcium oscillation. The majority of results presented in this review article have been published previously by Aizman *et al*.[5] Calcium (Ca^{2+}) is involved in the regulation of such diverse cellular processes as gene transcription, cell adhesion, cell growth, proliferation, and apoptosis.[6,7] Intracellular Ca^{2+} $[Ca^{2+}]_i$ oscillation may be the most versatile of all intracellular signals, since the cell can decode differences in the amplitude and frequency of these oscillations and translate them into specific cellular responses.[8,9] Low-frequency Ca^{2+} oscillations will specifically activate the transcription factor NF-κB by triggering proteolysis of the inhibitory subunit, IκB.[10,11] NF-κB plays an important role in regulation of cell growth, proliferation, and apoptosis.[12]

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Ann. N.Y. Acad. Sci. 986: 489–496 (2003).

METHODS

The majority of experiments were performed on primary cultures of rat proximal tubule cells (PTC), obtained from kidneys of 20-day-old Sprague-Dawley rats. These cells grow as clusters after 2–3 days in culture. At that time, they have a well-preserved phenotype and display the typical morphology of epithelial cells from proximal tubule. They have, as is typical for all renal tubule cells, a high level of Na,K-ATPase. In addition, rat PTC are particularly well suited for Ca^{2+} measurements with Fura-2AM.[13] Rat Na,K-ATPase has a relatively low ouabain sensitivity and full inhibition of the enzyme requires millimolar concentrations of ouabain.[14] Changes in Ca^{2+} concentration induced by ouabain/Na,K-ATPase complex were monitored by ratiometric fluorescent microscopy with Ca^{2+}-sensitive fluorophore, Fura-2AM. Frequency analysis was applied to determine specificity of the Ca^{2+} signal. To investigate the biological relevance of induced Ca^{2+} signal, we studied activation of the Ca^{2+}-dependent transcriptional factor, NF-κB. The interaction of Na,K-ATPase with IP_3R and its role in the generation of Ca^{2+} signals were explored by co-immunoprecipitation and fluorescence resonance energy transfer (FRET) analysis.

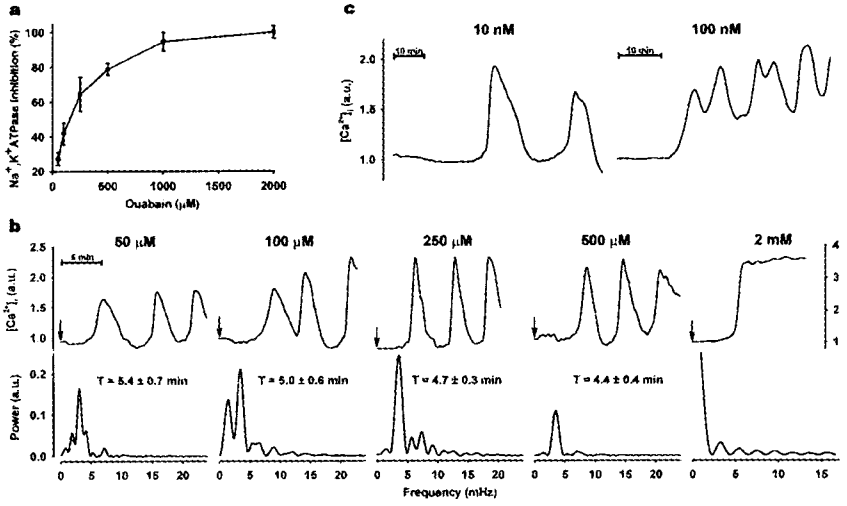

FIGURE 1. Effect of ouabain on $[Ca^{2+}]_i$ in primary culture of RPT cells. (**a**) Na,K-ATPase activity measured as ouabain-sensitive $^{86}Rb^+$ uptake (mean ± S.E.). (**b**) (*Upper panel*) Representative single cell $[Ca^{2+}]_i$ tracings in response to indicated ouabain concentrations. At time 0 (*arrow*), cells were exposed to ouabain concentrations ranging from 50 μM to 2 mM and recordings were made every 30 s. Arbitrary units (a.u.) represent ratio values corresponding to $[Ca^{2+}]_i$ changes. (*Lower panel*) Spectral analysis of ouabain-induced $[Ca^{2+}]_i$ oscillations. Each plot corresponds to the single cell recording above. $[Ca^{2+}]_i$ oscillation periodicity (T) of each ouabain concentration was calculated as mean ± S.E. from approximately 50 cells from at least three separate experiments. (**c**) Representative single cell $[Ca^{2+}]_i$ tracings observed in cells superfused for 3 hours at a slow rate (100 μL/min) with nanomolar ouabain. (From Aizman *et al.*[1] With permission from the National Academy of Sciences.)

RESULTS AND DISCUSSION

When PTC were exposed to ouabain in doses resulting in only partial Na,K-ATPase inhibition (10 nM–250 μM) (FIG. 1a), we observed slow, regular $[Ca^{2+}]_i$ oscillations (FIG. 1b). This response was detected in approximately one-third of the cells and was generally initiated in one cell at the periphery of a cell cluster and then propagated to neighboring cells. To determine to what extent the oscillations possessed an intrinsic regularity, we performed power spectrum analysis. Power spectrum analysis revealed a periodicity between 5.4 ± 0.7 min for 50 mM ouabain and 4.4 ± 0.4 min for 250 mM ouabain (FIG. 1b). The amplitude of the oscillations for all partially inhibitory ouabain doses was in the same range. A dose of 2 mM ouabain, which causes complete inhibition of rat Na,K-ATPase activity, did not cause oscillations, but resulted in a sustained increase in $[Ca^{2+}]_i$ (FIG. 1b).

It may be argued that Ca^{2+} oscillations were demonstrated in response to pharmacological doses of ouabain. *In vivo*, circulating levels of ouabain rarely exceed the picomolar-to-nanomolar range. However, it should be taken into account that ouabain binding to Na,K-ATPase is tight and long lasting—the "on-rate" for ouabain is ~20 times faster than the "off-rate."[15] Thus, per time unit, more ouabain molecules associate than dissociate with Na,K-ATPase. In tissue exposed to circulating blood, the number of Na,K-ATPase molecules occupied by ouabain will approach an equilibrium exponentially over time. In support of this, we demonstrated that when PTC cells were superfused with solutions containing ouabain in nanomolar range for more than an hour, Ca^{2+} oscillations were observed in 5% of the cells, while 30% responded at 250 μM ouabain (FIG. 1c). It should also be taken into account that the numbers of ouabain molecules bound to Na,K-ATPase must exceed a threshold to trigger a global cellular Ca^{2+} response.

To examine whether the Ca^{2+} oscillations were secondary to inhibition of Na,K-ATPase activity, we studied the effect of lowering extracellular K^+ concentration. Both ouabain and graded reduction of extracellular K^+ evoked similar, dose-dependent increases in intracellular Na ($[Na^+]_i$), indicating that both ouabain and low K^+ inhibited Na,K-ATPase activity to the same extent. However, lowering extracellular K^+ did not trigger calcium oscillations.[5]

The next set of studies was performed to elucidate the molecular mechanism of Ca^{2+} oscillations induced by the ouabain/Na,K-ATPase complex. In cells where the intracellular Ca^{2+} stores of the endoplasmic reticulum were depleted by preincubation with the sarco-endoplasmic reticulum ATPase (SERCA) pump inhibitor (20 μM cyclopiazonic acid), ouabain failed to induce Ca^{2+} oscillations. Regulated Ca^{2+} release from intracellular stores occurs via $InsP_3Rs$ or via ryanodine receptors (RyR). Stimulation of RyR with a low dose of ryanodine (10 μM) did not have any effect on $[Ca^{2+}]_i$. Inhibition of RyR by a higher dose of ryanodine (100 μM) did not prevent ouabain-induced $[Ca^{2+}]_i$ oscillations.[5] From this we conclude that RyR are not involved in ouabain-induced Ca^{2+} oscillations and that functioning RyR are of little, if any, importance in RPT cells. In contrast, IP_3 receptor inhibition with membrane-permeable IP_3R inhibitor 2-aminoethoxydiphenyl borate (2-APB) completely abolished the oscillations,[5] indicating that Ca^{2+} release via IP_3 receptor played a major role in Ca^{2+} oscillation induced by ouabain. Although 2-APB is the most specific membrane-permeable IP_3R inhibitor available today, it can also affect Ca^{2+} release–

activated Ca^{2+} channels (CRAC).[16] $InsP_3Rs$ are, either directly or via an anchor protein, coupled to CRAC channels, the function of which is essential for the maintenance of Ca^{2+} oscillations.[17,18] We conclude from these studies that release of calcium from intracellular stores via IP_3R is an essential contribution to the ouabain/Na,K-ATPase–induced calcium oscillations.

We also found that calcium-free media abolished ouabain-induced Ca^{2+} oscillations. Influx of Ca^{2+} from the extracellular space may, at least partially, occur via L-type voltage gated Ca^{2+} (L-VGC) channels, which are to a limited extent expressed in RPT cells[19] Two inhibitors of L-VGC channels, nifedipine and verapamil, both abolished the ouabain-induced Ca^{2+} oscillations, suggesting that L-VGC channels are involved in the generation of Ca^{2+} oscillations. However, both these inhibitors also exhibit significant antioxidant activity[20] and there is some evidence that they may also inhibit CRAC channels.[21] Therefore, it is possible that the observed inhibition of Ca^{2+} oscillations by nifedipine and verapamil is not solely a result of their effect on L-VGCC.

Since Na,K-ATPase is an electrogenic pump, it cannot be excluded that, even in epithelial cells, partial inhibition of its activity by ouabain may lead to some membrane depolarization and therefore may activate L-VGCC. Depolarization of cell membrane by the depolarizing agent, 4-aminopyridine (4-AP) or high extracellular $K^{+,}$ however, did not cause $[Ca^{2+}]_i$ oscillations.[5] Activation of L-VGCC by BayK 8644 (an L-type voltage-gated Ca^{2+} channel agonist; Sigma) causes a slow increase in intracellular Ca^{2+} but no oscillations. Taken together, these data show that activation of L-VGCC alone is not sufficient to trigger ouabain-induced Ca^{2+} oscillations.

The classical way to activate IP_3Rs involves increased generation of IP_3, triggered by ligand/G-protein coupled receptor interaction and PLC activation. Preincubation of cells with a PLC inhibitor, U73122, did not prevent ouabain-induced oscillations (unpublished observation). To test the efficiency of PLC inhibition, cells were also exposed to bradykinin, a well-known activator of phospholipase C (PLC) and $InsP_3$ production. Preincubation of RPT cells with a PLC inhibitor abolished bradykinin-induced Ca^{2+} transients. Taken together, these findings indicate that activation of PLC and subsequent increased generation of IP_3 are not required for induction of Ca^{2+} oscillations by ouabain/Na,K-ATPase complex.

A close spatial proximity between plasma membrane and endoplasmic reticulum has been demonstrated in renal epithelial cells.[22] It was therefore reasonable to hypothesize that IP_3R may interact with Na,K-ATPase. In ongoing studies we have found that Na,K-ATPase co-localizes with two subtypes of IP_3R (types 2 and 3). To further investigate the spatial relationship between Na,K-ATPase and $InsP_3R$ we are now using FRET, which provides resolution in the nanometer scale. The study is performed on COS cells, stably transfected with green fluorescent protein (GFP)-tagged rat Na,K-ATPase catalytic α1 subunit. Na,K-ATPase α-subunit fused to GFP on the NH_2-terminus (NKA-GFP) serves as a FRET donor. IP_3R types 2 and 3 labeled with Cy3-conjugated secondary antibody serve as FRET acceptor. According to the spectral properties, the combination of GFP and Cy3 allows detection of FRET at a distance up to approximately 12 nm.[23] So far we have observed a significant FRET effect between Na,K-ATPase and IP_3R types 2 and 3, indicating that Na,K-ATPase and $InsP_3R$ are separated by less than ~12 nm. Preincubation of cells with ouabain appears to enhance Na,K-ATPase/IP_3R FRET efficiency.

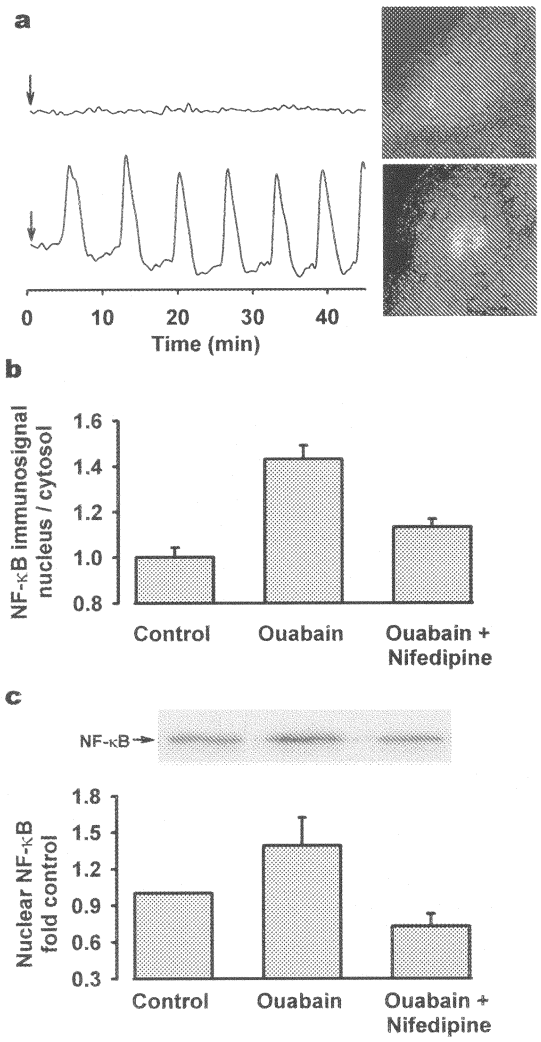

FIGURE 2. Effect of $[Ca^{2+}]_i$ oscillations on ouabain-induced NF-κB activation. (**a**) A cell cluster was treated with 250 μM ouabain (*arrow*) and individual cells were analyzed for both $[Ca^{2+}]_i$ and NF-κB immunofluorescence. *Upper panel* shows a typical nonoscillating $[Ca^{2+}]_i$ response (*left*) and its corresponding cellular NF-κB localization (*right*). *Lower panel* shows a typical oscillating $[Ca^{2+}]_i$ response (*left*) and its corresponding cellular NF-κB localization (*right*). (**b**) Semiquantitative analysis of NF-κB immunofluorescence signal showing translocation from cytosol to nucleus in cells exposed to 250 μM ouabain, in the absence or presence of 50 μM nifedipine. Values are mean ± S.E. 50–150 cells. Representative Western blot and densitometric analysis of 3–5 experiments showing changes in (**c**) nuclear NF-κB protein in cells exposed to 250 μM ouabain in the presence or absence of nifedipine. (From Aizman *et al.*[1] With permission from the National Academy of Sciences.)

Since both Na,K-ATPase and IP_3R are anchored by cytoskeleton proteins,[24,25] it was reasonable to expect that perturbation of the actin cytoskeleton network may influence the physical and/or functional Na,K-ATPase/IP_3R interaction. In fact, actin cytoskeleton hyperpolymerization or depolymerization by jasplakinolide (JP) or cytochalasin D (CytD), respectively,[26] abolished ouabain-induced Ca^{2+} oscillations.

We suggested a model for Ca^{2+} signaling triggered by ouabain/Na,K-ATPase complex. According to this model Na,K-ATPase and IP_3R interact with each other and this interaction requires an intact actin cytoskeleton. Ouabain, via allosteric changes in Na,K-ATPase, enhances the Na,K-ATPase/IP_3R interaction and triggers the frequency of the Ca^{2+} oscillations. Perturbations in actin cytoskeleton disrupt Na,K-ATPase/IP_3R interaction and thereby abolish Ca^{2+} oscillations. Ouabain-induced Ca^{2+} oscillations also require several permissive factors, such as Ca^{2+} influx via voltage-gated Ca^{2+} channels and/or CRAC channels.

The involvement of signaling cascades initiated by ouabain/Na,K-ATPase complex in the regulation of cell growth, proliferation and apoptosis have been previously suggested by several groups.[4,27,28] NF-κB is involved in the transcriptional regulation of many genes related to growth, differentiation, and apoptosis.[12] Additionally, NF-κB activation has been shown to be sensitive to and preferentially activated by slow Ca^{2+} oscillations.[10] This prompted us to study the effect of ouabain-induced Ca^{2+} oscillations on the activity of the transcription factor NF-κB. In unstimulated cells, NF-κB is predominantly located in the cytoplasm in association with inhibitory peptide IκB. Upon activation, NF-κB translocates to the nucleus.[12] The ratio between NF-κB immunosignal from the nucleus and from the cytosol has been semiquantitatively estimated (FIG. 2b). NF-κB nuclear staining was increased in cells that responded to ouabain with typical $[Ca^{2+}]_i$ oscillations (FIG. 2a). In cells where no effect of ouabain on $[Ca^{2+}]_i$ was detected, we did not observe any significant effect on the nuclear NF-κB immunosignals.[5] Subcellular fractionation and immunoblot studies the confirmed cytosolic–nuclear redistribution of NF-κB signals in all cells exposed to ouabain doses sufficient to trigger calcium oscillations (FIG. 2c). Interestingly, cells exposed to an ouabain concentration (2 mM) that caused a sustained increase in $[Ca^{2+}]_i$ exhibited a lesser degree of NF-κB activation.[5]

SUMMARY

We have demonstrated a novel role for Na,K-ATPase as a signal transducer involved in transcriptional regulation in mammalian cells. Taking into account the ubiquitous expression of Na,K-ATPase, it will be important to identify the cell-specific effects of this signaling. It will also be important to explore how the function of Na,K-ATPase as the key regulator of intracellular Na^+ and K^+ homeostasis may interrelate to its function as a signal transducer.

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